GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

Formerly G-33-H09 [x] ORIGINAL [ ] REVISION NO. _____

Project No. G-33-H02 (R6121-9A0) GTRC/GHX DATE 4 / 17 / 86 - Revis

Project Director: S.W. May; R. H. Felton School/GHX Chemistry

Sponsor: DHHS; PHS; NIGMS NIH

Type Agreement: Grant No. 5R01-GM23474-09

Award Period: From 4/1/86 To 3/31/87 (Performance) 6/31/87 (Reports)

Sponsor Amount:	This Change	Total to Date
Estimated:	$	$ 164,048
Funded:	$	$ 164,048

Cost Sharing Amount: $ 8,634 Cost Sharing No: G-33-314

Title: Non-Heme Metallo Oxygenase Catalysts

ADMINISTRATIVE DATA OCA Contact John B. Schonk

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Bethesda, MD

Defense Priority Rating: N/A Military Security Classification: N/A

(or) Company/Industrial Proprietary: N/A

RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval – Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with GIT

COMMENTS:

No funds may be expended after 3/31/87

The original initiation package is revised to reflect the correct project number. Previously distributed paperwork should be discarded.

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GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 6/24/88

Project No. G-33-H09 School/Lab Chemistry

Includes Subproject No.(s) N/A

Project Director(s) S. W. May/ R. H. Falton GTRC (GIT)

Sponsor DHHS/PHS/NIH/NIGMS

Title Non-Heme Metallo Oxygenase Catalysts

Effective Completion Date: 3/31/87 (Performance) N/A (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
- [XX] Final Invoice or Copy of Last Invoice Serving as Final FSR
- [ ] Release and Assignment
- [ ] Final Report of Inventions and/or Subcontract:
  Patent and Subcontract Questionnaire sent to Project Director [ ]
- [ ] Govt. Property Inventory & Related Certificate
- [ ] Classified Material Certificate
- [ ] Other

Continues Project No. Continued by Project No. G-33-H10

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Other

SECTION IV PROGRESS REPORT SUMMARY	GRANT NUMBER GM 23474	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR MAY, Sheldon W.	PERIOD COVERED BY THIS REPORT FROM	THROUGH
NAME OF ORGANIZATION Georgia Institute of Technology	4/1/86	3/31/87

TITLE *(Repeat title shown in item 1 on first page)*
Non-Heme Metallo Oxygenase Catalysis

(SEE INSTRUCTIONS)

PUBLICATIONS

"EXAFS of Non-Heme Iron Proteins" R.H. Felton, P.A. Morris, L.R. Furenlid, S.W. May and E.A.Stern, BNL Ann. Rept, 1986

"Structure and EXAFS of Diaquatetrakis(imidazole)cobalt(II) Dichloride", L.R. Furenlid, D.G. Vandervear, and R.H. Felton, Acta Cryst. C. 42 806, 1986

"EXAFS of an Enzyme Reaction Transient: The "ESO_2" of Protocatechuate 3,4-dioxygenase", R.H. Felton, S.W. May, J. Kaighobadi, L. Furenlid, P. Morris, and C. Oldham, Fed. Proceed. 46 in press.

"EXAFS of Non-Heme Iron Containing Proteins", P.A. Morris, Ph.D. Thesis, Georgia Institiute of Technology, 1986.

"Bromine as an EXAFS Marker: Substrate Binding in Protocatechuate 3,4-dioxygenase" in preparation.

"An Improved Isolation of Phenylalanine Hydroxylase Suitable for Large Scale Preparations", J.H. Han, A. Katopodis and S.W. May, in preparation.

"Preparation and properties of 6-bromophenylpterin", S.R. Sirimanne and S.W. May, in preparation

PROGRESS REPORT

Non-heme metallo oxygenases represent more than 80% of all known dioxygenases and a large number of monooxygenases, and thus a definition of the molecular basis of their catalytic action is highly relevant to many key biological processes. The broad objective of our program is to continue our analysis of the involvement of non-heme iron in the catalytic pathway of the bacterial dioxygenase, protocatechuate-3,4-dioxygenase (PCD), and to carry out comparative studies with the mamalian non-heme iron monooxygenase, liver phenylalanine hydroxylase (PAH), the mamalian coppper monooxygenase, dopamine-B-monooxygenase (DBM), and the bacterial non-heme iron monooxygenase, P. oleovorans epoxidase/hydroxylase (POEH).

The following paragraphs summarize our progress during this past year of our program.

Aromatic Dioxygenases, Transferrin and Model Complexes

Substrates and Inhibitors: During this past year, this part of our dioxygenase program focused almost entirely on working out the biochemical conditions necessary for building up and trapping the "ESO_2" transient species along the reaction pathway of the slow substrate, 3,4-dihydroxyphenylpropionate. We considered this of utmost priority, since successful EXAFS of this species would represent, to our knowledge, the first time this technique has ever been applied to a fully competent transient of a reaction pathway (as opposed to an inhibitor complex or a substrate complex lacking a reactant, such as ES for PCD). Together with our ongoing work on EI, ES, and E transition state analogs, results with ESO_2 would provide highly signiicant insight into the ligation changes at Fe during individual steps along the catalytic pathway.

A protocol was developed to monitor ESO_2 formation and decay under varying conditions of solvent and concentrations of reactants. The exceedingly high amounts of enzyme required for EXAFS is always a problem which must be overcome, but in this case the problem was confounded by the fact that a buildup of ESO_2 of 2-3 mM would be required for accurate EXAFS analysis, which is far in excess of the total solubility of oxygen in water at room temperature. After many trials, procedures were worked out to buildup ESO_2 under hyperbaric oxygen conditions (3-4 atm.) at 4°C, and to rapidly trap this species by freezing at -80°C. Kinetic methods were worked out to measure the germane rate constants and monitor formation and decomposition of the transients, in order to assure agreement with values previously determined by steady state and stopped flow analyses. Laborious, large-scale isolations were carried out to provide sufficient enzyme for these studies and for preparation of the sample which was actually subjected to EXAFS analysis in our December 1986 Brookhaven run (see below).

Other experiments in this phase of the program included preparation of crystals of PCD for solid state EXAFS measurements as a prelude to experiments were S and/or I will be diffused directly into such crystals. Also, careful reconstitution experiments on transferrin species were carried out in order to resolve some inconsistencies in our initial EXAFS reults with this protein.

EXAFS: The intensity and quality of the NSLS beam is markedly improved and has permitted us to collect XAFS on a number of samples.

As discussed above, "ESO_2" was trapped in an EXAFS cell at cryogenic temperature and its optical spectrum measured immediately prior to and following the EXAFS data collection by means of a specially constructed double beam fiber optic spectrometer. The spectra showed some alteration due to irradiation, which is plausibly assigned to ozinide formation in the O_2-rich sample. Upon thawing and further reaction, the characteristic ES spectrum was observed. This confirms that, upon thawing, the expected turnover of "ESO_2" indeed occurred, thus depeleting oxygen and trapping the PCD as ES due to the prsence of excess substrate. Since the solution was frozen, care was taken to move the sample slightly during data collection to reduce or remove Bragg peak scattering. Preliminary analyses showed the following: (1) loss of chelation by substrate or product, (2) a six-coordinate iron, similar to ES but in contrast to native PCD, (3) strong disorder of the first-shell relative to E or ES, (4) retention of histidyl ligation. First-shell analyses on E, ES, EI, and complexes with transition state analog is complete. The iron in native PCD and its complexes with 3-chloro-hydroxybenzoate (I) or TSA (TSA = 2-hydoxyisoniciotinic acid N-oxide) is five coordinate; in contrast, the iron in ES (S = protocatechuate, 5-bromoprotocatechuate, or dihydroxyphenylpropionate) is six coordinate. Average first-shell distances in E, ES, and EI are about 2.00 A, while E.TSA complexes possess average distances of 2.05 A.

Excepting the anomalous EI complex, all species show two histidyl ligands at the active site.

A collaboration with S. Mangani and I. Bertini (Italy) has been initiated on EXAFS studies of transferrin. Our interest is to understand in transferrin and PCD structural differences at the iron-containing site of the proteins, which contain very similar ligands at this site. We see clear differences in the first and third (His) shell.

In a preliminary investigation of the utility of EXAFS to examine crystalline enzyme complexes, we have obtained data on thermolysin and its complexes with the inhibitors, HSCH2-CH(CH2Bz)CO-Ala-Gly-NH2 and HONH-Bzm-Ala-Gly-OH. The data are of excellent quality and exhibit changes at the Zn due to binding. Upon completion of the analysis, comparsion will be made between EXAFS and X-ray crystallographic results.

Metallo-Monooxygenases: Phenylalanine Hydroxylase and Dopamine-B-Monooxygenase

As mentioned in last year's report, isolation of PAH on the scale required for EXAFS, as well as stability of the protein itself and of the Fe ligation environment, represent major quetsions which must be resolved if EXAFS is to be fruitfully applied to this protein. Many large scale isolations were carried out this past year using several variations of both the Kaufman and Shiman literature procedure. (We recently calculated that the total PAH isolated this past year exceeds the total of a decade of literature reports from the Kaufman lab!) Since for EXAFS purposes it is critical to remove any contaminating ferritin, we now utilize FPLC as a final purification step, and we routinely examine isolated PAH by electrophoresis.

Serious problems have been encountered in preparing PAH samples suitable for EXAFS. Typically, combined preps totalling about 50 - 60 mg of PAH are accumulated and then concentrated to volumes below 500 microliters in order to produce a final solution sufficiently concentrated for meaningful EXAFS (ca. 2 mM in Fe). With some samples of PAH this has caused cloudiness and precipitate formation. Of even greater concern is that while we take care to measure specific activity and to determine Fe concentration colorimetrically after purification and again after EXAFS, our December 1986 Brookhaven data indicated that only a fraction of the expected Fe was actually present in the sample. This is highly puzzling; it may indicate leakage of Fe during ultrafiltration, something totally inconsistent with statements in the literature. Moreover, there is possiblity that PAH becomes unstable at such extremely high concentrations. We are currently completing the prepration of new PAH and also attempting to reconstitute earlier preps which had previously been highly concentrated by ultrafiltration. We are scheduled to run these samples at Brookhaven on 19 January, 1987.

Preliminary EXAFS analysis on a 0.7mM (in iron) sample of PAH showed a first shell dominated by nitrogenous ligands with an average distance of 2.04 A.

As mentioned in our last report, very good progress has been made in preparation and characterization of modified phenylalanines and pterins. Alternate catalytic competencies for PAH will be investigated using our novel substrate analogs, vinyl-Phe and pMeS-Phe, as soon as our January EXAFS run is complete. A prototype of another novel class of compounds has been prepared as a potential mechanism-based inhibitor for PAH. The p-pyridine analog of Phe has been prepared and characterized, and we plan to synthesize the m- isomer as well. If PAH is capable of oxygenating such compounds to the corresponding pyridine N-oxides, these should be potent ligands for the Fe and may be excellent inhibitors. Moreover, the N-oxides are very useful EXAFS probes in investigating the question of whether the Fe is actually participating in substrate

oxygenation. Once again, these studies will occupy our attention during the coming year.

We now have in hand a vastly more efficient isolation method for dopamine-b-monooxygenase, which starts with isolation and purification of chromaffin cells. We calculate that one month of isolation work will provide more than enough DBM for EXAFS. We plan to run E and EI samples with DBM once beam time becomes available to us after the 5-6 month Brookhaven shutdown which commences in March, 1987.